

REMARKS

Claims 56 and 61 were pending. Claim 61 is amended herewith. Support for the amendment is found throughout the specification at, *inter alia*, page 14, lines 21-26. It is believed that the amendment adds no new matter. Claims 56 and 61 are pending, and no claim is allowed.

Applicants gratefully acknowledge the rejoining of claim 56 with claim 61.

Rejection under 35 U.S.C. § 102 (b) - Claim 61

Claim 61 remains rejected under 35 U.S.C. § 102 (b) over Kleinerman 1989 and Kleinerman 1992 for reasons of record. Briefly, the Examiner continues to assert that the “method of the prior art comprises the same method steps as claimed in the instant invention” and that the “claimed method is anticipated because the method will inherently lead to the amelioration of mucositis.” Applicants traverse this rejection.

Applicants respectfully submit that the Kleinerman references fail to anticipate the claimed invention because each and every element is not present in the cited references. More particularly, the method of using MTP-PE to ameliorate mucositis is not inherent in the disclosure of the Kleinerman references because the claimed method does not necessarily flow from the teachings of these references. In order to establish inherency, the references must make clear that the missing descriptive matter, *i.e.*, the use of MTP-PE to treat mucositis, is *necessarily* present in the thing described in the reference, and that it would be *so recognized* by persons of ordinary skill. See MPEP § 2112 (IV) (citation omitted). Neither Kleinerman reference meets these criteria.

A. The cited references and the knowledge provide no fact or technical reasoning that supports an assertion of inherency

The Kleinerman references fail to provide a basis in fact or/or technical reasoning to reasonably support the determination that the allegedly inherent amelioration of mucositis through the administration of L-MTP-PE in subjects treated with an anti-neoplasia agent *necessarily* flows from its teachings of the applied prior art.

First, the prior art teaches that MTP-PE has biological properties likely to exacerbate mucositis. It was already well-known in the art at the time of filing that MTP-PE up-regulated the expression of inflammatory cytokines such as TNF- α and IL-1 in human monocytes. *See e.g.*,

Exhibit B at 1034. Inflammation mediated at least in part by TNF- α was known to be the first phase in mucositis. *See* Exhibit A (reviewing the current knowledge regarding the pathophysiology of mucositis).¹ Briefly, Sonis describes mucositis as a complex biological process which occurs in four phases: 1) an inflammatory/vascular phase, 2) an epithelial phase, 3) an ulcerative/bacteriological phase, and 4) a healing phase. *See* Exhibit A at 40. According to Sonis, TNF- α and IL-1 mediate the inflammatory/vascular phase of mucositis mediated in part by causing tissue damage and inciting an inflammatory response resulting in increased subepithelial vascularity, respectively. *See id.*

Data reported in Kleinerman 1992 provides only further support for a role in MTP-PE in inducing an inflammatory response that elicits mucositis. For example, three of the five cases (cases 1, 2, and 3) demonstrated increased vascularity in the tumor nodules after L-MTP-PE administration. Additionally, in four of five cases (cases 1, 2, 3, and 5), Kleinerman reports increased infiltration by chronic inflammatory cells in four of the five cases examined. *See* Kleinerman 1992 at 214-18. While these changes could be beneficial for their anti-tumor effects (*see* Kleinerman 1992 at 219), the same changes in mucosal epithelia are likely to worsen the inflammatory/vascular phase of mucositis given the art-recognized role of vascularity in mucositis pathology. Taken together, the cited references and the knowledge in the art suggests that the same effect of L-MTP-PE that aids in fighting tumors could, if seen in the mucosa, would actually exacerbate mucositis.

Furthermore, the art also discloses MTP-PE as a proliferative stimulator of intestinal epithelial cells. *See e.g.*, Killion et al., *Cancer Biother. Radiopharmaecut.* 11: 363, 370 (1996) (already of record). Stimulation of epithelial cell proliferation was believed to actually increase the susceptibility of these cells to the toxic effects of chemotherapy. *See* Exhibit C at 753-54. In point of fact, Sonis suggested the inhibition or reduction of the rate of mucosal proliferation as an effective approach to the prevent mucositis, citing studies where the administration of mitotic agents exacerbated mucositis when administered prior to receiving chemotherapy. *See id.* at 755. Again,

¹ Applicants note that the publication of the Sonis coincides with the priority date of the instant application, and therefore is a reflection of the state of the art at the time of filing.

in view of such knowledge, a person of skill in the art would not recognize the use of MTP-PE to treat mucositis as inherent in the Kleinerman references.

Therefore, the objective evidence fails provide a basis in fact or/or technical reasoning to support the Examiner's assertion of inherency, and in fact teaches away from the claimed methods. The Kleinerman references and the knowledge in the art lacks any disclosure that makes clear that the use of MTP-PE to treat mucositis is inherent in the cited disclosures. Indeed, a person of skill in the art would recognize only that MTP-PE would likely exacerbate mucositis, not ameliorate or prevent mucositis as in the claimed methods.

B. A person of skill in the art would not recognize the use of MTP-PE to treat mucositis as a “missing element” in the Kleinerman references.

The combination of an absence of any express disclosure in the Kleinerman references regarding the treatment of mucositis and the art-recognized role for inflammation in mucositis pathology fails to provide a credible basis to support an assertion that a person of skill in the art would recognize the Kleinerman references as teaching the use of MTP-PE to mucositis. To date, the Examiner has provided no objective evidence that a person of skill would recognize Kleinerman 1989 as including each and every claim limitation of the instant methods. Applicants respectfully request that if the Examiner's rejection is based on facts within his personal knowledge, the Examiner will support this rejection with those facts in an affidavit by the Examiner according to MPEP § 2144.03. According to MPEP § 2144.03,

When a rejection is based on facts within the personal knowledge of the examiner, the data should be stated as specifically as possible, and the facts must be supported, when called for by the applicant, by an affidavit from the examiner.

C. Kleinerman 1989 fails to anticipate the instant methods

Again, Applicants **strongly** disagree with the characterization of the teachings of the Kleinerman publication, in particular Kleinerman 1989. Kleinerman 1989 fails to teach or suggest mucositis as a target for MTP-PE treatment to one of ordinary skill in the art of cancer biology. Kleinerman 1989 simply discloses the *in vitro* cytotoxicity and cytokine production profile of peripheral monocytes in patients receiving MTP-PE. This is not sufficient to anticipate the instant method. Kleinerman neither expressly nor implicitly addresses the treatment of any side effect, or

mucositis in particular, resulting from a neoplastic agent. Moreover, Kleinerman lacks any indication that patients with mucositis were included in the patient cohort. In the absence of such disclosure, the current scientific knowledge in the art fails to support the assumption that the patients inherently have mucositis for reasons of record. *See* Response filed March 1, 2004. Thus, it cannot reasonably be considered inherent that such patients were included in the study.

D. The instant claims are clearly distinguishable from *Ex parte Novitski*

Because the element of MTP-PE as a treatment option for mucositis is missing from the Kleinerman references, *Ex parte Novitski* is readily distinguishable. For the reasons discussed above, L-MTP-PE's mucositis-ameliorating activity cannot be said to be inherently disclosed in the Kleinerman references or recognized in the art. In view of the knowledge in the art and the teachings of the Kleinerman references, a person of ordinary skill would recognize only that L-MTP-PE may exacerbate mucositis. It is not enough to allege that the prior art includes examples of L-MTP-PE activity that suggest L-MTP-PE's potential to ameliorate mucositis only in hindsight using the guidance provided by the instant specification, particularly when the art clearly teaches away from that the claimed use. Inherency may not be established by mere probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is simply not sufficient to establish inherency. *See* MPEP § 2112 (IV). Unless the relevant determination—that L-MTP-PE possesses mucositis-ameliorating properties—can be shown to *necessarily* flow from the teachings of the prior art, inherency is not present, and *Ex parte Novitski* is inapplicable.

In view of the above, Applicants submit the basis of the rejection may be removed.

Rejection under 35 U.S.C. § 102 (b) - Claim 56

Claim 56 is rejected under 35 U.S.C. § 102 (b) as being anticipated by Kleinerman et al., *J. Clin. Oncol.*, 9:259-67 (1991). Citing *Ex parte Novitski*, the Examiner asserts that the “method of the prior art comprises the same method steps as claimed in the instant invention” and thus “the claimed method is anticipated because the method will inherently lead to the amelioration of mucositis in the subset of those patients that develop mucositis.” Applicants traverse this rejection.

Kleinerman 1991 fails to anticipate the claimed invention because it lacks each and every element of the claimed methods. MPEP §§ 2131-2131.01. Kleinerman 1991 is merely an *in vitro* study and does not include any clinical data in human patients. Most importantly, Kleinerman 1991 is completely silent regarding mucositis. Therefore, the Examiner must again rely on the inherency of the use of MTP-PE to treat mucositis. As with the Kleinerman references discussed above, there is no basis in fact or/or technical reasoning to reasonably support the determination that the allegedly inherent amelioration of mucositis through the administration of L-MTP-PE in subjects treated with an anti-neoplasia agent *necessarily* flows from its teachings of the Kleinerman 1991 reference.

For the reasons discussed above, a person of skill in the art would not recognize that MTP-PE as a treatment for mucositis. Moreover, in the complete absence of any discussion whatsoever of mucositis, a credible assertion cannot be made that the Kleinerman 1991 reference makes clear that the missing element of treatment with mucositis with MTP-PE necessarily flows from the disclosure in the cited reference. As discussed in early responses, mucositis is not inherently present in all patients. Thus, no objective evidence has yet been provided that a person of skill would recognize Kleinerman 1991 as including each and every claim limitation of the instant methods or that such a teaching necessarily flows from the Kleinerman 1991 reference. Applicants respectfully request that if the Examiner's rejection is based on facts within his personal knowledge, the Examiner will support this rejection with those facts in an affidavit by the Examiner according to MPEP § 2144.03. According to MPEP § 2144.03,

When a rejection is based on facts within the personal knowledge of the examiner, the data should be stated as specifically as possible, and the facts must be supported, when called for by the applicant, by an affidavit from the examiner.

In view of the above, Applicants submit the basis of the rejection may be removed.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 204372000901. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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Mucositis as a biological process: a new hypothesis for the development of chemotherapy-induced stomatotoxicity

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Abstract

Mucositis induced by antineoplastic drugs is an important, dose-limiting and costly side effect of cancer therapy. The ulcerative lesions which result are frequent systemic portals of entry for microorganisms which inhabit the mouth and consequently are often sources of systemic infection in the myelosuppressed patient. A number of clinical observations and the inconsistency of responses to a broad range of treatment modalities suggests a physiological complexity to mucositis which has not previously been comprehensively considered. We now propose a hypothesis as to the mechanism by which mucositis develops and resolves, which is based on four phases: an initial inflammatory/vascular phase; an epithelial phase; an ulcerative/bacteriological phase; and a healing phase. The role of cytokines as initiators and amplifiers of the process is discussed, as is the potential influence of genetic factors in establishing risk and modifying the course of stomatotoxicity. © 1998 Elsevier Science Ltd. All rights reserved.

1. Introduction

Mucositis induced by antineoplastic drugs is an important, dose-limiting and costly side effect of cancer therapy. The ulcerative lesions produced by stomatotoxic chemotherapy are painful, restrict oral intake and, importantly, act as sites of secondary infection and portals of entry for the endogenous oral flora [1]. The overall frequency of mucositis varies and is influenced by the patient's diagnosis, age, level of oral health and the type, dose and frequency of drug administration [2]. Some degree of mucositis occurs in approximately 40% of patients who receive cancer chemotherapy [2]. About one-half of those individuals develop lesions of such severity as to require modification of their cancer treatment and/or parenteral analgesia. The condition's incidence is consistently higher among patients undergoing conditioning therapy for bone marrow transplant, continuous infusion therapy for breast and colon cancer and therapy for tumours of the head and neck. Among patients in high risk protocols, severe mucositis occurs with a frequency in excess of 60% [3-5].

As a consequence, it is not unusual for mucositis to necessitate a de-escalation of a planned dosing regimen. Because of the concomitant neutropenia which often

occurs secondary to chemotherapy-induced myelosuppression, mucositis is a significant risk factor for systemic infection. Patients with mucositis and neutropenia have a relative risk of septicemia that is greater than four times that of individuals without mucositis [6].

In addition to its impact on quality of life and morbidity and mortality, mucositis also has a significant economic cost. For example, in patients undergoing autologous bone marrow transplant for haematological malignancies, the length of hospital stay among patients with mucositis is 5 days longer than patients without the condition [7]. At an average day rate of \$4,500 for this patient population, this results in additional charges of \$22,500 per patient.

A number of clinical observations suggest a physiological complexity in the development of mucositis. There is great variability in the stomatotoxicity of treatment regimens [8]. Patient age affects risk: younger patients develop mucositis more frequently than older patients receiving the same form of treatment for similar malignancies [2].

Once lesions develop, they heal more quickly in the younger population. Patient diagnosis affects risk as patients with haematological malignancies are more likely to develop lesions than are patients with solid

tumours [2]. Concomitant radiation significantly enhances the stomatotoxic potential of chemotherapy. Patients who receive total body irradiation as part of a conditioning regimen for BMT develop mucositis of an intensity and frequency that exceeds patients who receive only chemotherapy [4,5]. The status of the patient's oral health is a well-established modifier [9,10]. Patients in good dental health who maintain scrupulous oral hygiene during cancer treatment tend to have fewer episodes of mucositis than do patients with poor oral health and maintenance. Finally, is the observation that patients of the same age, having the same tumour, receiving the same dose and form of chemotherapy and with equivalent oral status do not develop mucositis at the same frequency.

The development of effective treatment or the prevention and elimination of mucositis has been elusive. More than 50 published studies exist which document clinical investigations aimed at the palliation, prevention or reduction of stomatotoxicity. The range of medications that have been used for a mucositis indication is extensive and includes topical antimicrobials [11,12], marrow-stimulating cytokines [13-16], vitamins [17], inflammatory modifiers [18-21], palliative rinses [22], amino acid supplements [23], cryotherapy [24,25] and laser treatment [26]. While a lack of a standardised assessment scale confounds interpretation of outcomes [8,27-29], the analysis of treatment modalities suggests an inconsistency of response that is often hard to reconcile relative to the mechanism by which mucositis occurs. Similarly, results of animal studies in which different cytokines and antimicrobials have been tested have also, at times, been puzzling.

Analyses of mucositis has been largely based on observational data. While there have been suggestions as to the mechanisms whereby mucositis develops, for the most part, the pathophysiology of the condition is undefined. Although different potential therapeutic agents sometimes modified outcome, they did so in a way that was not always reproducible or consistent [30,31]. We now propose a hypothesis as to the mechanisms by which mucositis develops and heals which is based on animal and clinical data, but is to some degree still speculative.

Mucositis is a complex biological process which occurs in four phases (Fig. 1):

1. inflammatory/vascular phase;
2. epithelial phase;
3. ulcerative/bacteriological phase;
4. healing phase.

Each phase is interdependent and is the consequence of a series of actions mediated by cytokines, the direct effect of the chemotherapeutic drug on the epithelium, the oral bacterial flora and the status of the patient's bone marrow. As demonstrated by observations in

models of graft versus host disease, injury to host tissues elicited by radiation and/or chemotherapy is capable of causing the release of cytokines from the epithelium and connective tissues [32,33]. Chemotherapy, in particular, affects the release of both interleukin-1 (IL-1) and tumour necrosis factor- α from the epithelium [34]. Ionising radiation, at doses which in themselves are not directly damaging to tissue, also causes the release of these cytokines from the epithelium and connective tissues [35]. Tumour necrosis factor is capable of causing tissue damage [36] and may be an accelerating and initiating event in the mucositis process. IL-1 incites an inflammatory response resulting in increased sub-epithelial vascularity [37] with a potential consequent increase in the local levels of cytotoxic agent.

It is likely that this response is relatively acute. Early sequential histological data obtained from mice treated with bleomycin or 5-fluorouracil (5-FU) demonstrate more cellularity of the subepithelial tissue, vascular dilation, and leukocyte margination only 24 h after drug administration [38]. The inflammatory/vascular response is probably not as specific to certain classes of chemotherapeutic agents as is the epithelial phase of therapy. In addition, the concomitant use of radiation and chemotherapy is likely to amplify and prolong the release of cytokines and thereby exacerbate tissue response.

The epithelial phase is probably the best documented. Dividing cells of the oral mucosal epithelium are non-specifically affected by many antineoplastic agents [39]. It is apparent that not all cancer chemotherapeutic drugs are equally active in this role; those drugs which affect DNA synthesis (the S phase of the cell cycle) seem to be the most efficient.

Thus, antimetabolites, such as methotrexate, 5-FU and cytarabine, which are cell cycle, phase specific agents affecting the S phase, are more stomatotoxic than are drugs which are cycle phase non-specific drugs. Support for the hypothesis that epithelial basal cell damage leads to mucositis comes from clinical and experimental observations. Children, who typically have a higher proliferating fraction of basal cells are three times more likely to develop mucositis than are elderly adults in whom the basal cell proliferative rate has slowed [40]. The administration of epidermal growth factor to animals prior to the administration of 5-FU markedly increases the incidence of mucositis, most likely by increasing the rate of basal cell proliferation and thereby sensitising the cells to the stomatotoxic effects of chemotherapy [41]. Finally, temporarily taking basal cells out of cycle with transforming growth factor- β 3 appears to be stomatoprotective [42,43], as does modification of apoptotic cell death [44].

The epithelial phase may be the most profound in terms of the production of ulcerative lesions. Reduced epithelial renewal results in atrophy and typically begins

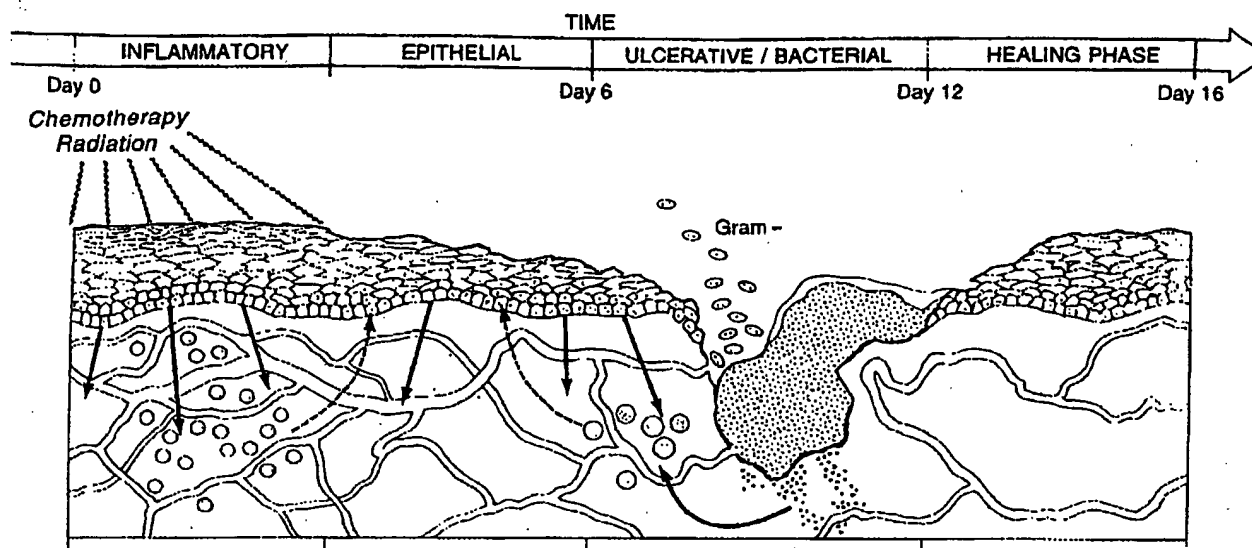


Fig. 1. The four phases of mucositis. The times indicated may vary slightly. Phase 1. Inflammatory/vascular phase. Shortly after the administration of radiation or chemotherapy cytokines are released from the epithelial tissue. These include tumour necrosis factor- α , interleukin-1 and perhaps, interleukin-6. Ionising radiation also incites cytokine release from the adjacent connective tissue. It is likely that these cytokines cause local tissue damage as the initiating event in the development of mucositis. Increased vascularity caused by IL-1 may result in additional concentrations of cytotoxic drug in the mucosa. Increased submucosal cellularity is evident at this stage. Phase 2. Epithelial phase. Both radiation and chemotherapy, especially with drugs affecting the S phase of the cell cycle, impact on the dividing cells of the oral basal epithelium, resulting in reduced epithelial renewal, atrophy and ulceration. The latter is most likely exacerbated by functional trauma and amplified by a flood of locally produced cytokines. Phase 3. Ulcerative/bacterial phase. The ulcerative phase is the most symptomatic and perhaps the most complex. Localised areas of full-thickness erosions occur which often become covered by a fibrinous pseudomembrane. Secondary bacterial colonisation of the lesion occurs with a mixed flora, including many gram negative organisms, providing a source of endotoxin (lipopolysaccharides) which further stimulate cytokine release from connective tissue borne around the cells. These cytokines, plus nitric oxide, serve to intensify the patient's condition. Importantly, from the stand-point of overall morbidity, the ulcerative phase generally occurs at the time of the patient's maximum neutropenia. Phase 4. Healing. The healing phase consists of a renewal of epithelial proliferation and differentiation, normalisation of the peripheral white blood cell count and re-establishment of the local microbial flora.

about 4–5 days after drug administration. It is initially synchronous with the inflammatory/vascular phase (G. Shklar, personal communication). It is probable that the marked erythema noted in many chemotherapy recipients [45] represents a combination of increased vascularity and reduced epithelial thickness. In addition, a flood of locally produced cytokines may amplify tissue destruction. Once the tissue becomes atrophic and its renewal is inhibited, functional trauma leads to ulceration.

The ulcerative phase is the most symptomatic and perhaps the most biologically complex phase of mucositis, as it presents the opportunity for both intrinsic and extrinsic factors to interact. Additionally, it is the time at which mucositis has the greatest potential impact on the patient's well-being. By the time ulceration is clinically apparent, typically about 1 week after the administration of the drug, early evidence of neutropenia is notable.

The severity of neutropenia progresses to a nadir, usually 14 days after the initiation of therapy and about 3 or 4 days after peak mucositis [46,47]. Bacterial colonisation of mucosal ulceration is a common finding leading to local secondary infection and, as previously noted, a microbiological reservoir for a systemic influx of

organisms. Importantly, the oral flora of neutropenic patients differs from that of the healthy population in that it is rich in gram negative organisms, in addition to typical alpha-haemolytic streptococci [48].

The result is a flow of endotoxin (lipopolysaccharides) into submucosal tissue where it is likely to interact with tissue-borne mononuclear cells to cause the release of additional IL-1 and TNF and the production of nitric oxide [49], all of which may play an amplifying role in the patient's local mucosal injury.

It is important to note that it is quite possible that a role exists for transcription factors which modify the genetic expression of cytokines and enzymes which are critical in producing tissue damage [50]. Such factors, such as NF-kappa B, increase the rate of gene transcription and thereby the rate of messenger RNA and protein production [51].

While environmental modification of transcription factor expression has been described, the impact of chemotherapy has yet to be investigated. In addition, it seems possible that genetic influences on inflammatory response might offer at least a partial explanation for the variance in patient response to antineoplastic therapy.

The final phase of mucositis is that which is related to healing, and includes elements related to cell proliferation

and differentiation, normalisation of peripheral blood white cells and control of the local bacterial flora. The rapidity with which this phase proceeds affects the duration of the condition, but probably not peak intensity. Any factor which negatively impacts on wound healing will undoubtedly affect this phase.

The hypothetical mechanism proposed here for mucositis development and healing attempts to correlate a variety of clinical and laboratory findings into a comprehensive picture of the condition. It seems likely that additional detail will result as more research is done in this area. Importantly, this model offers a variety of therapeutic opportunities which could be directed at any of the four phases of the condition. With the increased use of aggressive chemotherapeutic regimens, the importance of mucositis as a limiting toxicity is escalating, making its control an important priority in clinical oncology.

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Liposomal Muramyl Tripeptide Up-Regulates Interleukin-1 α , Interleukin-1 β , Tumor Necrosis Factor- α , Interleukin-6 and Interleukin-8 Gene Expression in Human Monocytes¹

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ABSTRACT

Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) is a new biologic agent presently in clinical trials for metastatic osteosarcoma and melanoma. The mechanism of L-MTP-PE antitumor activity is linked to its activation of monocyte tumoricidal function. The purpose of this study was to determine whether L-MTP-PE affected the expression of cytokine genes in monocytes. Monocyte interleukin (IL)-1 α , IL-1 β , IL-6, IL-8 and tumor necrosis factor (TNF)- α expression were all up-regulated after a 2-h incubation with L-MTP-PE. The increased expression of IL-1 α , IL-1 β , IL-6 and IL-8 persisted up to 72 h. Increased TNF- α expression declined by 24 h. The

kinetics of cytokine expression stimulated by L-MTP-PE were different from those seen after lipopolysaccharide (LPS) stimulation. Lipopolysaccharide stimulation caused a rapid increase in cytokine expression followed by a rapid decline. L-MTP-PE did not affect the expression of these cytokines in lymphocytes, nor did L-MTP-PE upregulate IL-2 expression in lymphocytes. The early up-regulation of all five cytokines was due to an increase in the transcriptional activity. Modification of mRNA stability was not detected at 2 h but was seen after a 24-h exposure to L-MTP-PE. The subsequent production and secretion of these cytokine proteins may play a role in L-MTP-PE antitumor activity.

MTP-PE is a synthesized lipophilic analog of muramyl dipeptide, the smallest component of the mycobacterium capable of stimulating the immune system (Schroit and Fidler, 1982). MTP-PE has potent monocyte/macrophage-activating properties (Kleinerman *et al.*, 1983a). L-MTP-PE was specifically designed for *in vivo* targeting to macrophages by intravenous infusion and is the only form of the drug currently available for clinical trials (CGP 19835A Lipid).

The *in vivo* administration of L-MTP-PE in mice with B16 melanoma metastases resulted in the activation of alveolar macrophages to the tumoricidal state and in tumor regression of lung and lymph node metastases (Fidler *et al.*, 1981; Poste *et al.*, 1982). L-MTP-PE efficacy against canine osteosarcoma has also been demonstrated (MacEwen *et al.*, 1989). Because of its success in preclinical animal studies with melanoma and osteosarcoma, phase I and II trials using L-MTP-PE are now under way in patients with these malignancies (Fujimaki *et al.*, 1993b; Kleinerman *et al.*, 1989, 1992a,b; Liebes *et al.*, 1992; Murray *et al.*, 1989; Urba *et al.*, 1990). We have reported

elevations in plasma TNF, IL-6 and IL-1 after L-MTP-PE administration (Kleinerman *et al.*, 1992a; Murray *et al.*, 1989). We have further demonstrated that monocytes produce and secrete TNF and IL-1 after exposure to L-MTP-PE (Maeda *et al.*, 1991). We now demonstrate that L-MTP-PE up-regulates IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA expression in peripheral blood monocytes at a transcriptional level. L-MTP-PE had no effect on the expression of these cytokines in lymphocytes.

Materials and Methods

Reagents and drugs. Roswell Park Memorial Institute medium 1640, Hanks' balanced salt solution without Ca⁺⁺ or Mg⁺⁺, fetal bovine serum and L-glutamine were purchased from Whittaker Bioproducts, Inc. (Walkersville, MD). Lymphocyte separation medium was purchased from Organon Teknika (Durham, NC). All agents were free of endotoxin, as determined by the Limulus amoebocyte lysate assay (sensitivity limit, 0.025 ng/ml). *Salmonella typhosa* LPS was purchased from Sigma Chemicals Co. (St. Louis, MO). L-MTP-PE and free MTP-PE, supplied by Ciba-Geigy, Ltd. (Summit, NJ), were prepared as previously described (Fujimaki *et al.*, 1993a; Maeda *et al.*, 1991). L-MTP-PE (500 nM) is equivalent to 1.67 μ g/ml.

Molecular probes. The cDNA probes for human IL-1 α , a 460-bp

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Abbreviations: MTP-PE, muramyl tripeptide phosphatidylethanolamine; L-MTP-PE, liposome MTP-PE; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; bp, base pairs; GM-CSF, granulocyte-macrophage colony-stimulating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, sodium chloride/sodium μ citrate; SDS, sodium dodecyl sulfate.

EcoRI-BamHI insert and human IL-1 β , a 530-bp *BamHI-Nde I* insert, were obtained from Dr. U. Gubler (Hoffman-La Roche, Inc., Nutley, NJ). The human TNF- α cDNA, an 800-bp *EcoRI-EcoRI* fragment, was obtained from Genentech, Inc. (South San Francisco, CA). Human IL-2 probe was a 1000-bp *PstI-PstI* insert. Human IL-8 probe was a 500-bp *EcoRI-EcoRI* insert. Human GM-CSF cDNA, a 200-bp *EcoRI-Bgl II* insert, was kindly provided by Dr. R. Kurzrock (M. D. Anderson Cancer Center, Houston, TX). Human IL-6 RNA probe was made by cutting a pGEMl-based plasmid with *HindIII* (1200 bp cDNA) and was obtained from Dr. S. Gillis (Immunex Research and Development Corporation, Seattle, WA). Chicken β -actin cDNA was a 1800-bp *PstI-PstI* insert. Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA was a 1300-bp *PstI-PstI* insert.

Purification of normal human monocytes, lymphocytes and mononuclear leukocytes. Mononuclear leukocytes were separated by density gradient centrifugation using lymphocyte separation medium from normal human buffy coats as previously described (Fujimaki *et al.*, 1993a; Maeda *et al.*, 1991). The mononuclear leukocytes were then fractionated by elutriation in a Beckman J 6 M (Beckman Instruments, Inc., Fullerton, CA) to purify the monocyte and lymphocyte fractions (Bakouche *et al.*, 1987). The purity of the elutriated monocyte and lymphocyte fractions was >95% as confirmed by Diff-Quik staining (American Scientific Products, McGaw Park, IL) and nonspecific esterase stain. The purified monocytes, lymphocytes and in some cases the unfractionated mononuclear leukocytes were cultured in Roswell Park Memorial Institute medium 1640 medium supplemented by 5% fetal bovine serum, 1% L-glutamine, and 1% gentamycin.

RNA extraction and Northern blot analysis. Because adherence has been previously shown to upregulate IL-1, TNF, IL-6, and IL-8 expression, the purified monocytes, lymphocytes and mononuclear leukocytes were incubated in Falcon 2006 polypropylene tubes (Becton Dickinson Labware, Lincoln Park, NJ) at 5×10^6 cells at 37°C (Donnelly *et al.*, 1991). For RNA extraction, a total of 25×10^6 cells were incubated with culture medium in the presence or absence of L-MTP-PE, free MTP-PE or LPS. Total RNA was prepared by the acid guanidine isothiocyanate-phenol-chloroform extraction method as previously described (Fujimaki *et al.*, 1993a; Maeda *et al.*, 1991). After quantitation by spectrophotometry, equivalent amounts of RNA (20 μ g/lane) were size fractionated by electrophoresis in 0.1% agarose gels (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD) containing 6.6% formaldehyde (Sigma). RNA was then blotted by overnight capillary transfer onto nylon transfer membranes (Micron Separations, Inc., Westboro, MA). The membranes were UV cross-linked, prehybridized and then probed with the ³²P-labeled human IL-1 α , IL-1 β , TNF α , IL-8, GM-CSF, IL-2, chicken β -actin, and rat GAPDH cDNA probes. The membranes were washed with a 0.1 x SSC, 0.1% SDS solution three times. ³²P-labeled probes were produced using a random hexamer labeling kit from Amersham (Arlington Heights, IL). The specific activity was between 1×10^9 and 2×10^9 cpm/ μ g DNA. The human IL-6 probe was radiolabeled as previously described (Melton *et al.*, 1984). The probe-specific activity was greater than 10^8 cpm/ μ g. These membranes were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at -70°C. For kinetics analysis, scanning density was determined with Personal Densitometer (Molecular Dynamics, Sunnyvale, CA), and values were normalized for differences in GAPDH-scanning densities. After normalization, the density of each time point was calculated by subtracting the density in control cells at the same time point. Percent of the maximum expression for each time point (% maximum expression) was calculated as follows: % maximum expression = calculated density of each time point/peak density seen for that cytokine $\times 100$.

Nuclear run-on transcription assay. After various stimulations, 5 to 7×10^7 monocytes were washed with cold phosphate-buffered saline and with Earle's balanced salt solution (Whittaker) and suspended with hypotonic buffer (20 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 10 mM NaCl, 0.5 mM dithiothreitol, 0.3 M sucrose, 0.25% Nonidet P-40) on ice for 5 min. The detergent-treated monocytes were then layered onto an equal volume of isolation buffer (20 mM Tris/HCl, pH 7.5, 5

mM MgCl₂, 10 mM NaCl, 0.5 mM dithiothreitol, 0.8 M sucrose) and centrifuged at $500 \times g$ for 10 min. The supernatant was aspirated, and the pelleted nuclei were gently resuspended in 125 μ l of transcription buffer (10% glycerol, 20 mM HEPES, pH 7.8, 1 mM MgCl₂, 2 mM MnCl₂, 142 mM KCl), 0.25 μ M of CTP, ATP, GTP, 1.25 μ M of dithiothreitol, 0.75 μ M of spermidine, 1 μ l of RNasin (Promega Biotech, Madison, WI) and 100 μ Ci of [α -³²P]UTP (Amersham) and incubated at 30°C for 30 min with gentle shaking. The reaction mixture was treated with 12.5 μ g of RQ1 RNase-free DNase (Promega Biotech) and incubated at 30°C for 5 min. Proteinase K (100 μ g), 4 μ l of 0.2 M EDTA, 17.5 μ l of 10% SDS and 20 μ g of yeast tRNA were then added. The mixture was incubated at 40°C for 45 min, extracted with phenol/chloroform, precipitated in ethanol/sodium acetate and the pellet dissolved and precipitated in 10% trichloroacetic acid, 30 mM sodium pyrophosphate and 1 mM UTP. Finally, the mixture was precipitated in ethanol/sodium acetate. The nuclear run-on transcript was resuspended in 500 μ l of hybridization buffer (50% formamide, 6 x SSPE, (1 x SSPE is 0.15M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA, pH 7.4), 5 x Denhardt's solution, 0.1% SDS, 200 μ g/ml of denatured salmon sperm DNA) and hybridized with DNA-immobilized filters at 44°C for 5 days. The procedure allowed the incorporation of 0.1 to 0.4×10^6 cpm (total) into 5 to 7×10^7 isolated nuclei.

DNA immobilized filters were prepared as follows: 20 μ g of linear DNA with target sequence was denatured with 0.8 M NaOH for 30 min at room temperature. The filters were neutralized with 200 μ l of 10 x SSC, and the denatured DNA solution was filtered immediately through nylon filter membrane and UV cross-linked. After hybridization, the filters were washed three times with 0.1% SDS, 2 x SSC and 2 mM EDTA at room temperature for 30 min and then washed three times with 0.1% SDS, 0.1 x SSC and 2 mM EDTA at 50°C for 30 min. The filters were exposed at -70°C for 7 to 10 days. Scanning density was determined as above and values were normalized for differences in β -actin scanning densities. Relative transcriptional activity is calculated as follows: relative transcriptional activity = density of the specific probe/ β -actin density on the same filter.

Analysis of mRNA half-life. Elutriated normal human monocytes were incubated with 1.67 μ g/ml of L-MTP-PE for 2, 8 and 24 h. After incubation with L-MTP-PE, the monocytes were washed to remove the stimulant, and then actinomycin D (Sigma) at 10 μ g/ml in culture medium was added. At indicated times (0, 30, 60, 120 min), total RNA was extracted as previously described. Scanning density was determined as described above.

IL-6 and IL-8 protein assay. IL-6 and IL-8 proteins were measured by enzyme-linked immunosorbent assay (T Cell Sciences, Cambridge, MA; R & D Systems, Minneapolis, MN). The cultured supernatants from monocytes incubated with L-MTP-PE (5×10^6 monocyte/ml) were collected and centrifuged.

Results

Dose response of L-MTP-PE. To determine the ideal amount of L-MTP-PE to use in our experiments, human monocytes were first treated with different concentrations of L-MTP-PE (fig. 1). L-MTP-PE (16.7 and 166 ng/ml) did not alter IL-1 β mRNA expression compared with that of control cells; however, both 1.67 and 16.7 μ g/ml increased the level of IL-1 β mRNA at 4 h. Because 1.67 and 16.7 μ g/ml both appeared to reproducibly up-regulate cytokine expression, we used 1.67 μ g/ml of L-MTP-PE in all subsequent experiments.

Kinetics of increased cytokine expression stimulated by L-MTP-PE. To determine how rapidly L-MTP-PE up-regulated cytokine mRNA levels, monocytes were incubated with L-MTP-PE for 1 to 72 h. Monocytes incubated with L-MTP-PE had increased levels of IL-1 α , IL-1 β , IL-6, IL-8 and TNF α mRNA by 2 h compared with levels in control cultures (fig. 2). The increased expression of IL-1 α , IL-1 β , IL-6 and IL-

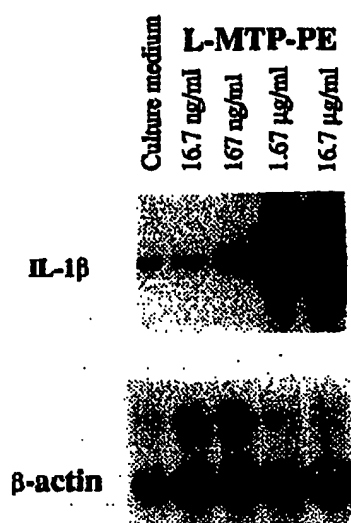


Fig. 1. Dose response of L-MTP-PE. Human mononuclear leukocytes were incubated with different concentrations of L-MTP-PE for 4 h. RNA was extracted and analyzed by Northern blot using a 32 P-labeled IL-1 β and β -actin probe.

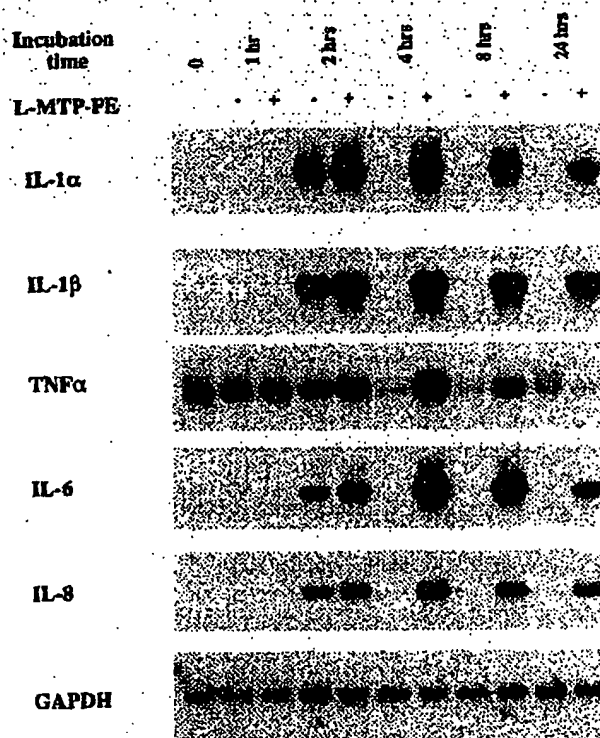


Fig. 2. Expression of IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA in monocytes after incubation with L-MTP-PE. Human monocytes from a single donor were isolated by elutriation and incubated with 1.67 μ g/ml of L-MTP-PE for 0 to 24 h. RNA was extracted and analyzed by Northern blot using 32 P-labeled cytokine probes (one of five experiments).

8 persisted for up to 72 h, but TNF α expression declined more rapidly and did not persist after 24 h. We were unable to detect any change in IL-2 or GM-CSF mRNA after the incubation of monocytes with L-MTP-PE for 1 to 72 h (data not shown). As compared with LPS stimulation (1 μ g/ml), the increased expression of each cytokine was sustained longer after L-MTP-PE stimulation. Exposing monocyte to LPS caused a rapid increase in cytokine expression after a rapid decline. Seen after

L-MTP-PE, the increase in mRNA expression by LPS was not sustained (fig. 3). Cytokine mRNA kinetics stimulated by free MTP-PE (1.67 μ g/ml) were almost the same as kinetics by L-MTP-PE except early up-regulation of each cytokines mRNA (IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA were up-regulated by 1 h; data not shown).

Increased levels of IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA were also detected when mononuclear leukocytes, as opposed to purified monocytes, were incubated with L-MTP-PE (data not shown). We were unable to detect any change in cytokine mRNA expression when purified lymphocytes were incubated with L-MTP-PE for 1 to 72 h. An example (one of three representative experiments) is shown in figure 4. L-MTP-PE also did not up-regulate lymphocyte IL-2 expression (data not shown). This was not an unexpected finding because liposomes must be phagocytized by the cell in order for the MTP-PE to be delivered. Lymphocytes are not phagocytic. We therefore concluded that the increase in cytokine mRNA seen in L-MTP-PE-treated mononuclear leukocytes was mainly secondary to L-MTP-PE effect on the monocyte population.

Effect of L-MTP-PE on cytokine nuclear transcription. To determine whether these increased levels of IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA were related to an increase in the transcriptional activity of these genes, we compared their transcription rates in nuclei isolated from L-MTP-PE-treated and untreated monocytes. We chose two different incubation times, 4 and 24 h. As shown in figures 5 and 6, the transcriptional rates of IL-1 β , TNF α , IL-6 and IL-8 were all increased in monocytes incubated with L-MTP-PE for 4 h compared with control monocytes incubated with medium for 4 h. At 24 h (figs. 5 and 6), the transcriptional rates of IL-1 β and IL-8 were still increased whereas the IL-6 and TNF α transcriptional rates were equal to those of the control monocytes. The transcription rate of IL-1 α was marginally elevated at both 4 and 24 h. We therefore concluded that the up-regulation of cytokine expression stimulated by L-MTP-PE involved increased transcription of these cytokine genes and that this appears to be an early event. IL-1 β , IL-6 and IL-8 stimulation is more pronounced than IL-1 α and TNF α stimulation, and the increased transcription of IL-1 α , IL-1 β and IL-8 persists longer than those of the other two cytokines.

Effect of L-MTP-PE on cytokine mRNA stability. To determine whether the increase in cytokine mRNA accumulation seen after L-MTP-PE stimulation was due in part to its effect on RNA half-life, mRNA stability assays were performed. Purified monocytes were incubated with L-MTP-PE for 2 and 24 h (time when increased mRNA accumulation is evident), and then 10 μ g/ml actinomycin D was added. At different times after actinomycin D treatment, total RNA was extracted, submitted to Northern blot analysis and quantified by densitometric scanning (fig. 7). L-MTP-PE treatment did not significantly modify the half-life of the IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA at 2 h; however, treatment of monocytes with L-MTP-PE for 24 h prolonged the half-life of each cytokine (fig. 7). Thus, the activity of L-MTP-PE at the 24 h time point may be indirect *via* the stabilization of the mRNA, as well as, direct *via* increased transcription. We concluded that prolonged L-MTP-PE incubation increased IL-1 α , IL-1 β , TNF α , IL-6 and IL-8 mRNA stability. The stability of β -actin mRNA was not modified by L-MTP-PE treatment (data not shown).

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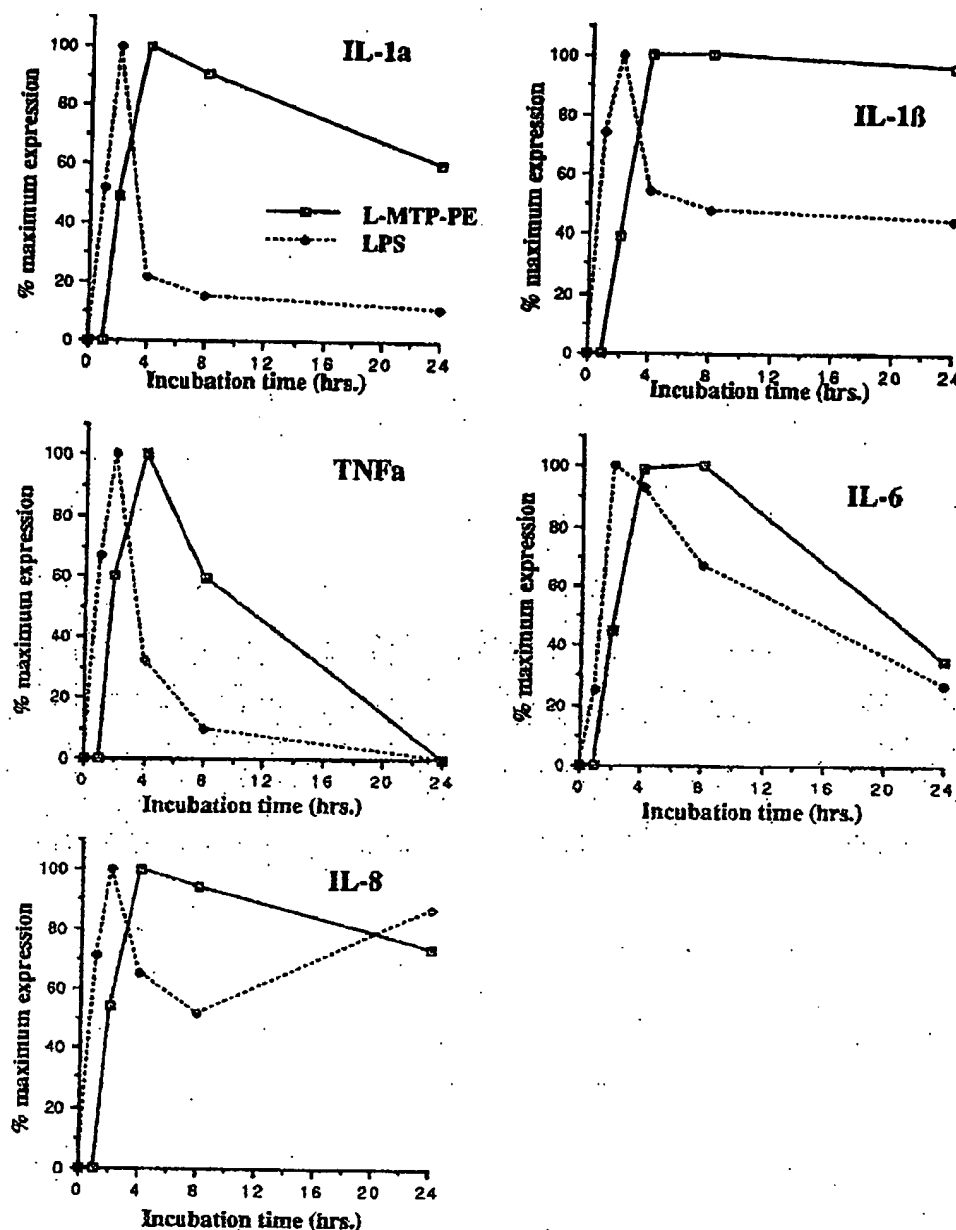


Fig. 3. Cytokine mRNA expression kinetics in monocytes stimulated by L-MTP-PE and LPS. Human monocytes from a single donor were incubated with L-MTP-PE (1.67 $\mu\text{g}/\text{ml}$) or LPS (1 $\mu\text{g}/\text{ml}$) for various times. RNA was extracted and analyzed by Northern analysis as described in figures 1 and 2. The density of each time point was normalized by GAPDH density and calculated by subtracting the density seen in control cells at same time point. The values of % maximum expression were then calculated as described under "Materials and Methods".

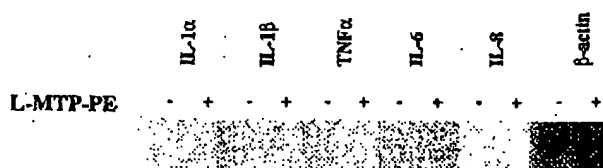


Fig. 4. Expression of cytokines in lymphocytes after stimulation with L-MTP-PE. Lymphocytes were isolated by elutriation, then incubated with L-MTP-PE for 8 h. RNA was extracted and analyzed by Northern blot as described in figure 2 (one of three experiments).

IL-6 and IL-8 secretion from L-MTP-PE-treated monocytes cultured supernatant and patient plasma. We have previously demonstrated that L-MTP-PE stimulated monocytes to produce IL-1 α , IL-1 β and TNF protein (Maeda *et al.*, 1991). Because IL-6 and IL-8 mRNA expression are

increased by L-MTP-PE, we wished to determine whether IL-6 and IL-8 proteins were also produced by monocytes in response to L-MTP-PE. Human monocytes were therefore incubated for 1 to 72 h with L-MTP-PE. Cultured supernatants were collected and assayed for IL-6 and IL-8 using an enzyme-linked immunosorbent assay. IL-6 protein was first detected after 4 h of stimulation with L-MTP-PE, and its production continued to increase for up to 72 h (table 1). Elevations in IL-8 protein secretion were seen as early as 1 h, with a dramatic increase between 2 and 4 h (table 1). TNF, IL-1 and IL-6 elevations in plasma have been demonstrated after L-MTP-PE infusion (Kleinerman *et al.*, 1992a; Murray *et al.*, 1989). To determine whether IL-8 was also induced after L-MTP-PE therapy, we monitored plasma IL-8 levels in five cancer patients receiving L-MTP-PE. Increased plasma IL-8 was demonstrated in four of five patients 2 to 3 h after L-MTP-PE infusion (fig. 8).

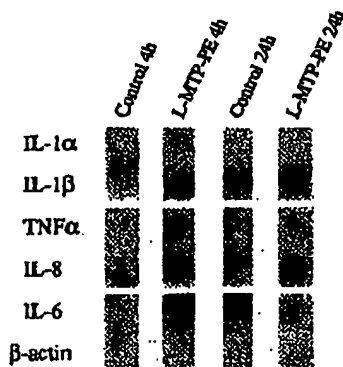


Fig. 5. Effect of L-MTP-PE on cytokine nuclear transcription. Nuclei were isolated from monocytes incubated with or without L-MTP-PE for 4 or 24 h. Run-on transcription assays were performed as described under "Materials and Methods".

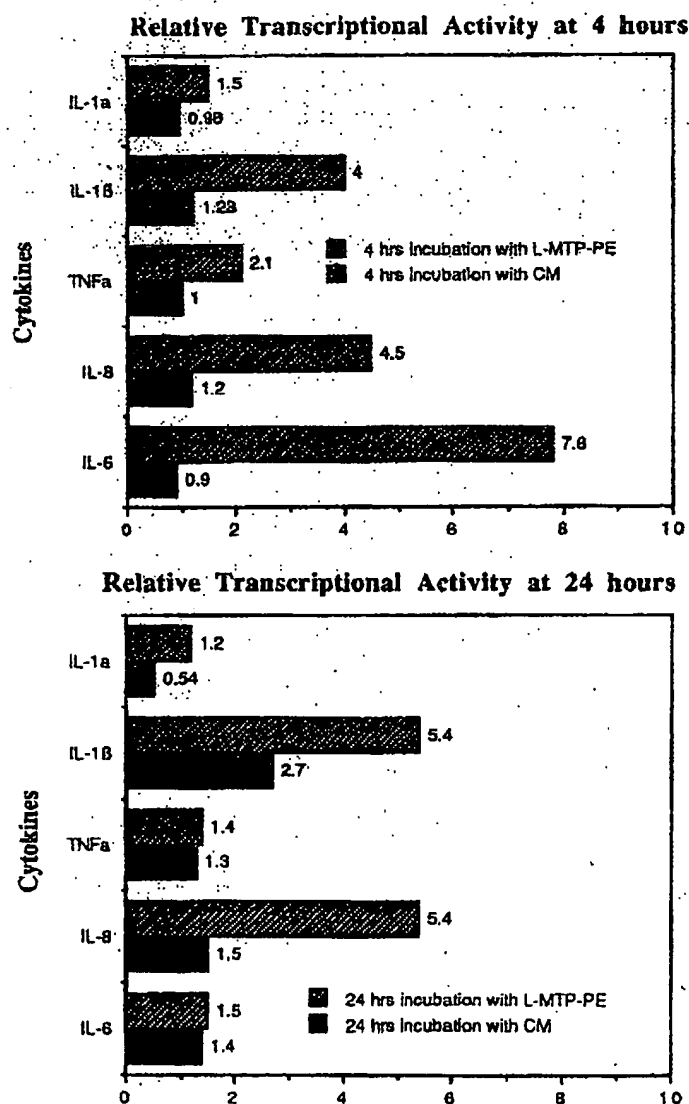


Fig. 6. Summary of transcription assay. Relative transcriptional activity between cells incubated with L-MTP-PE vs. culture medium was calculated by normalization of β -actin density.

Discussion

The present study demonstrated that the expression of several cytokines is upregulated in human monocytes by L-MTP-PE. These cytokines include IL-1 α , IL-1 β , TNF- α , IL-6 and IL-8. L-MTP-PE did not up-regulate the expression of these cytokine genes in lymphocytes, however. The observed increase in IL-1, TNF- α , IL-6 and IL-8 expression was not due to an overall activation of the monocytes, because L-MTP-PE did not modulate GM-CSF or IL-2 expression. Our results further suggest that the initial increase in each cytokine mRNA was due to increased transcription with no modification of the cytokine mRNA stability. However, at the later time points (8–24 h), L-MTP-PE exerted its effect by increasing both the transcription and the half-life of the specific cytokine mRNA.

We have previously demonstrated that L-MTP-PE stimulated monocytes to produce IL-1 and TNF proteins (Maeda et al., 1991). We now extend these results to show that L-MTP-PE also stimulated the production of IL-6 and IL-8 proteins. Because MTP-PE is a byproduct of the mycobacterium cell wall, it is perhaps not surprising that it can up-regulate IL-6 and IL-8 expression in addition to that of IL-1 and TNF. LPS has also been shown to induce the expression and protein production of these cytokines; however, the cytokine mRNA expression kinetics of monocytes stimulated with L-MTP-PE was distinct from those seen after LPS stimulation (fig. 3). The finding that L-MTP-PE maintained its stimulatory capacity is novel. Other investigators have demonstrated that when LPS was encapsulated into liposomes, it lost its ability to induce protein kinase C translocation (Bakouche et al., 1992) and IL-1 β protein production (Bakouche et al., 1987). Encapsulation also prevented the interaction of the hydrophobic portion of the lipid A component of LPS with the specific monocyte plasma membrane structures involved in activation of the cell (Dijkstra et al., 1987). By contrast, liposome-encapsulated MTP-PE was effective at stimulating both cytokine expression and protein production.

Liposomes are phagocytized by human monocytes within 30 min. Peak uptake occurs by 1 h (Kleinerman et al., 1983b). The modulation of cytokine expression occurred 1 h after the peak uptake time (2 h). If we allow for degradation of the liposome wall and for release of MTP-PE intracellularly, the time frame of increased expression (1 h) is similar to that observed with free LPS (fig. 3) or free MTP-PE (data not shown). L-MTP-PE had no direct effect on lymphocytes, presumably because there was no uptake of the liposomes. However, our observation of enhanced IL-6 expression and the subsequent production of IL-6 protein may suggest that L-MTP-PE can indirectly influence lymphocyte function when administered *in vivo*. IL-6 is a critical cytokine in the acute inflammatory response. IL-6 stimulates B cell maturation and proliferation, activates T-cell function and stimulates cytotoxic T cells (Kishimoto, 1989). We have demonstrated that IL-6 levels in plasma dramatically increase within 3 to 4 h after i.v. infusion of L-MTP-PE (Kleinerman et al., 1992a). Our present data suggest that this increase in plasma IL-6 may be secondary to L-MTP-PE ability to up-regulate monocyte production of IL-6. IL-6 has been shown to have antitumor activity in both a fibrosarcoma and a B16 melanoma model (Mule et al., 1992; Mullen et al., 1992; Sun et al., 1992). Tumor cells transduced with the IL-6 gene exhibited increased immunogenicity and decreased metastatic potential (Mullen et al., 1992). Infiltration of the IL-6-trans-

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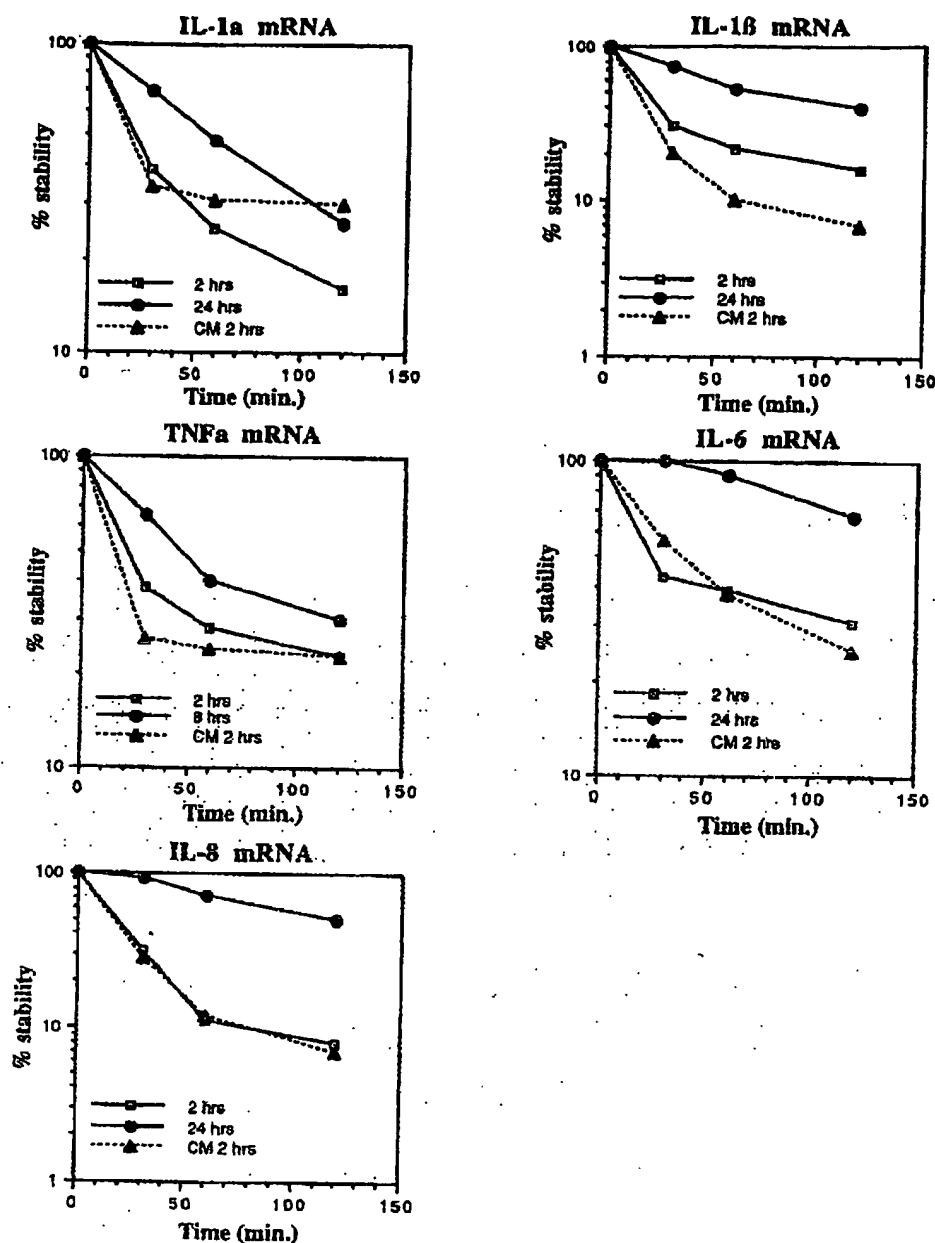


Fig. 7. Cytokine mRNA half-life in L-MTP-PE-stimulated monocytes. Human monocytes were isolated by elutriation and stimulated for the indicated time with L-MTP-PE (2, 8, 24 h) or control medium (CM, 2 h). Actinomycin D (10 μ g/ml) was then added, and total RNA was extracted at various times and analyzed by Northern blot. Autoradiography was scanned and the cytokine level expressed in percentage; 100% was assigned to the cytokine mRNA level after the L-MTP-PE incubation and immediately before adding actinomycin D (one of three experiments).

TABLE 1

L-MTP-PE-Induced IL-6 and IL-8 protein secretion

Monocytes (5×10^6) were incubated with 1.67 μ g/ml of L-MTP-PE for the indicated time. Cultured supernatants were collected and assayed for IL-6 and IL-8 using an enzyme-linked immunosorbent assay.

Incubation Time	IL-6	IL-8
h	pg/ml	ng/ml
0	0	0.85
1	0	2.8
2	0	60
4	47	5200
8	315	5200
16	1550	4600
24	1625	4400
48	1730	4300
72	1850	4300

ected tumor with host inflammatory cells was described (Sun *et al.*, 1992). Pulmonary tumors removed from osteosarcoma patients after L-MTP-PE therapy also demonstrated chronic inflammatory cell infiltration not typically observed in tumors removed from patients after chemotherapy or even in tumors from patients who have had no therapy (Kleinerman *et al.*, 1992b). Peripheral fibrosis around the tumor lesion was also observed (Kleinerman *et al.*, 1992b). We hypothesized that the fibrosis was caused by IL-1 and TNF secreted by pulmonary macrophages activated by L-MTP-PE. IL-6 may also have contributed to the inflammatory cell infiltration seen after L-MTP-PE therapy.

The production of IL-8 is also part of the inflammatory response. Neutrophil exposure to IL-8 results in neutrophil chemotaxis and degranulation (Baggiolini *et al.*, 1989; Van Damme *et al.*, 1988). Hence, L-MTP-PE therapy may also indirectly lead to neutrophil activation and recruitment into

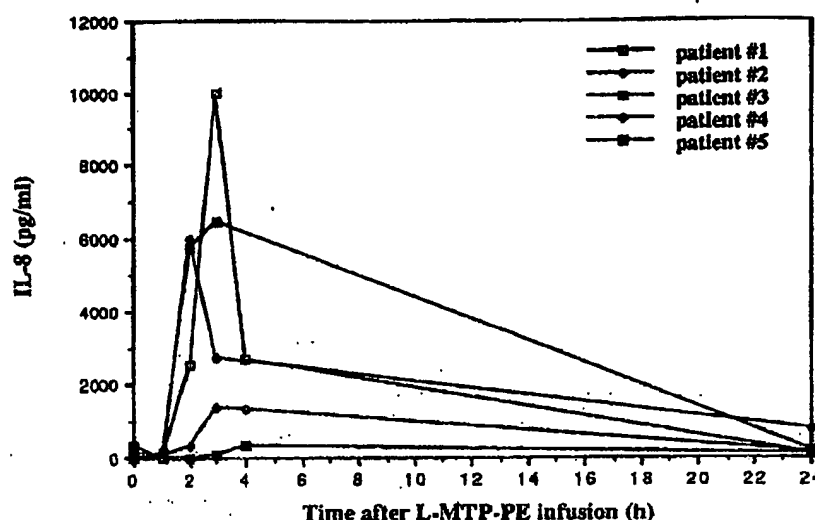


Fig. 8. Plasma levels of IL-8 after i.v. administration of L-MTP-PE. Blood samples were collected from five patients at various times after L-MTP-PE infusion. Plasma was separated, frozen and subsequently assayed for IL-8.

the tumor area. Although neutrophils were not observed in the inflammatory cell infiltration of the osteosarcoma lesions, we cannot exclude the possibility that the early recruitment of neutrophils into the tumor area contributed to the fibrosis formation around the tumor. Because surgery was performed 2 to 6 weeks after cessation of L-MTP-PE (Kleinerman *et al.*, 1992b), neutrophils may have exited the pulmonary circulation by the time of surgery.

Although additional *in vivo* studies are needed to determine whether any of these cytokines are indeed crucial to L-MTP-PE mechanism of action, the observation that L-MTP-PE up-regulates IL-1 α , IL-1 β , TNF, IL-6 and IL-8 indicates that the agent stimulates an inflammatory response. We therefore conclude that in addition to activating macrophage-mediated tumor cell killing, L-MTP-PE stimulates the production of inflammatory cytokines that serve to amplify the monocyte response by recruiting and activating other immune cells.

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Effect of epidermal growth factor on ulcerative mucositis in hamsters that receive cancer chemotherapy

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BRIGHAM & WOMEN'S HOSPITAL, HARVARD SCHOOL OF DENTAL MEDICINE, AND AMGEN CORPORATION

Ulcerative mucositis is a common, bothersome, and dose-limiting complication of cancer chemotherapy. It has been hypothesized that mucosal susceptibility to the degenerative effects of stomatotoxic drugs is related to the renewal rate of the buccal epithelium. This study was undertaken to evaluate the effect of epidermal growth factor, a molecule known to stimulate epidermal cell division, on the course, frequency, and healing of ulcerative mucositis in an animal model. Golden Syrian hamsters were subjected to a standard mucositis-induction protocol with 5-fluorouracil. Osmotic pumps were implanted into a space between the retractor muscle and the platysma cervical muscle, and delivered epidermal growth factor or placebo at a constant rate for 7 or 14 days. Epidermal growth factor increased oral mucosal breakdown in the face of antineoplastic therapy. The course and extent of mucositis was influenced by the timing of epidermal growth factor pump placement relative to the initiation of stomatotoxic therapy. These results support the hypothesis that the epithelial basal cell rate is one of the key elements in determining mucosal sensitivity to cancer chemotherapy.

(ORAL SURG ORAL MED ORAL PATHOL 1992;74:749-55)

Ulcerative oral mucositis is one of the most common, bothersome, and dose-limiting complications of cancer chemotherapy.¹⁻⁴ Because the oral mucosa undergoes frequent replication, it is especially sensitive to the direct cytotoxic effects of chemotherapy. Histologically, signs of oral mucosal atrophy become evident soon after therapy has begun. Inhibition of DNA replication of basal epithelial cells and cytolysis result in a thin, atrophic mucosa that is highly susceptible to spontaneous or traumatic ulceration. It has been hypothesized that the rate of basal epithelial cell proliferation affects the susceptibility of the oral mucosa to the stomatotoxic effect of chemotherapy.^{1, 5, 6}

Epidermal growth factor (EGF) is a 53-amino acid

polypeptide (mol. wt. 6000) first isolated from extracts of mouse submaxillary glands. The molecule has a number of activities including the induction of precocious eyelid opening and incisor eruption when injected into newborn mice.⁷ This response is attributed to stimulation of epidermal growth and keratinization. In addition, EGF directly stimulates the proliferation of basal epithelial cells in skin organ culture systems.⁸

Other mitogenic effects of EGF have been noted in vitro with a variety of cell types. In vivo studies in neonatal mice by Steidler and Reade⁹ suggest that subcutaneous injections of EGF cause an increase in epithelial thickness and keratinization of skin and oral mucosa. Recent studies have shown that EGF promotes proliferation and differentiation of the gastrointestinal mucosa.^{10, 11}

The mechanism of action EGF exerts after it binds to its receptor is currently under investigation. It is known that the EGF receptor is a glycoprotein with

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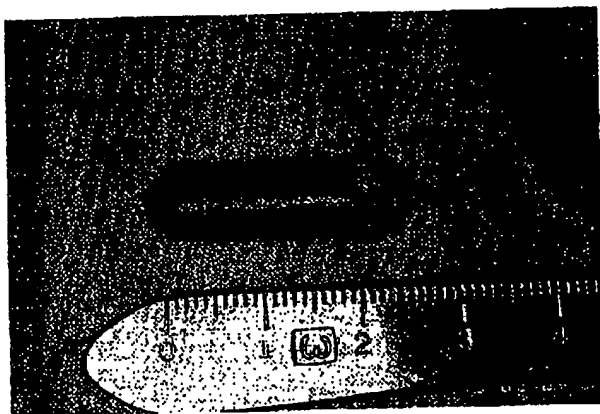


Fig. 1. Alzet osmotic pump filled with EGF ($8.33 \mu\text{g}/\mu\text{l}$) or placebo. Pump measures 3.0 cm in length and 0.7 cm in diameter.

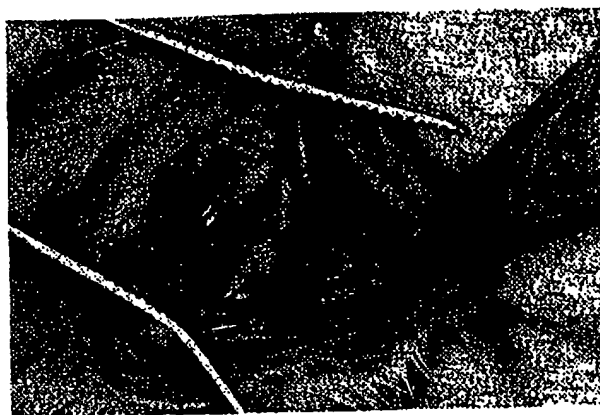


Fig. 2. Alzet osmotic pump sutured in place between retractor muscle and platysma cervicale muscle. Blunted cannula can be seen protruding through mucosa.

a molecular weight of 175,000. On binding, EGF is able to stimulate an intrinsic tyrosine kinase activity that autophosphorylates the receptor.¹² However, little is known about how the binding and autophosphorylation influence transduction of a mitogenic signal to the nucleus so as to alter gene regulation.

Because EGF stimulates epithelial proliferation, we investigated its effect, when applied topically, on the frequency, course, and healing of ulcerative mucositis. We hypothesized that early exposure of the oral mucosa to EGF simultaneous to stomatotoxic cancer chemotherapy would enhance the development of ulcerative mucositis. We also hypothesized that its effect during healing might be favorable in reestablishing intact epithelium.

MATERIAL AND METHODS

Animals

Male Golden Syrian hamsters, 8 to 10 weeks old, were obtained from Charles River Laboratories, Wilmington, Mass. Animals were kept five to a cage and provided with free access standard hamster food and water.

Epidermal growth factor

Human recombinant EGF was provided by Amgen Corp., Thousand Oaks, Calif., and loaded into 200 μl Alzet osmotic pumps (Alza Corp., Palo Alto, Calif.).

Pump placement protocol

To ensure uniform topical EGF delivery, an implanted osmotic pump protocol was devised. After induction, animals were anesthetized with ethyl ether; sodium pentobarbital (Nembutal) (50 mg/kg) was administered by intraperitoneal injection. The left buccal pouch was everted and an incision made at the

most superior aspect of the pouch in the mucobuccal fold. With blunt dissection a space was created between the retractor muscle and the platysma cervicale muscle. The pump, with a short contiguous cannula, was inserted and a purse-string suture was placed (Figs. 1 & 2). The cannula was allowed to protrude through the surgical site by about 3 mm. The end of the cannula was blunted to prevent mucosal irritation and dislodgement. Preliminary studies performed in our laboratory with dye-loaded pumps demonstrated their efficacy in delivering a uniform dose of material during a prolonged period. No infection or soft tissue anomalies were associated with pump placement. Pumps placed for 2 weeks delivered EGF at the rate of $0.5 \mu\text{l}/\text{hr}$; 1-week pumps delivered EGF at the rate of $1.0 \mu\text{l}/\text{hr}$.

Mucositis was induced in 60 hamsters by an established protocol.¹³ Briefly, hamsters received 5-fluorouracil (5-FU) (60 mg/kg) on days 0 and 5, with superficial irritation of the buccal pouch mucosa on day 7. Severe mucositis developed in all animals with this technique. Animals were randomly and equally divided into the following groups:

- Group 1:* On day 3 a 7-day pump containing EGF was implanted ($8.33 \mu\text{g}/\mu\text{l}$)
 - Group 2:* On day 7 a 7-day pump containing EGF was implanted ($8.33 \mu\text{g}/\mu\text{l}$)
 - Group 3:* On day 6 a 7-day pump containing EGF was implanted ($8.33 \mu\text{g}/\mu\text{l}$)
 - Group 4:* On day 1 a 14-day pump containing EGF was implanted ($8.33 \mu\text{g}/\mu\text{l}$)
 - Group 5:* On day 7 a 7-day pump containing placebo was implanted (control for group 2)
 - Group 6:* On day 1 a 14-day pump containing placebo was implanted (control for group 4)
- Animals were observed and weighed daily and had

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Table I. Mean mucositis score and standard deviation by day*

Day	Group					
	1	2	3	4	5	6
10	8.39	4.28	6.60	7.11	1.55†	6.78
	1.25	1.03	1.02	2.85	0.52	1.79
11	8.21	3.47	6.50	8.32	2.55	8.03
	1.65	2.43	1.06	1.62	2.08	2.09
12	9.39	7.63	7.94	8.14	NP	6.94
	0.56	1.63	1.20	1.27		1.74
13	NP	7.55	9.40	7.13	6.17	6.19
	NP	1.27	0.76	2.48	3.02	2.39
14	9.54	8.14	7.60	5.43	6.47	5.71
	0.90	0.84	2.76	3.38	2.63	1.76
15	8.67	8.78	6.08	4.13	5.56†	1.68
	2.10	0.97	0.88	3.77	3.02	0.62
16	9.63	6.64	6.42	3.46	4.56	0.75‡
	0.53	2.46	0.63	3.11	3.34	0.75
17	4.00	5.25	4.67	2.00	3.25	0.64
	0.00	2.90	2.10	3.11	1.89	0.67
18	6.00	4.43	5.58	2.45	3.38	1.71
	0.00	2.69	1.46	1.58	3.34	0.89
19	1.75	3.25	3.75	1.75	2.06	1.89
	0.00	2.92	1.89	0.88	1.91	1.38
20	4.00	4.13	4.83	1.35	2.75	2.00
	0.00	3.03	1.04	1.28	2.60	1.61

NP, Photographs not available for these time points.

*Mean mucositis scores for each group on each day. Top value in each row indicates mean mucositis score ranging from 0 to 10. Bottom value represents SD for that average score for each group on each day. Parametric statistical analysis was performed between groups 2 and 5 and groups 4 and 6 by unpaired *t* test.

†Statistically significant ($p < 0.01$).

‡Statistically significant ($p < 0.05$).

free access to food and water. Beginning on day 10 left buccal pouches were photographed daily with a Yashica Dental Eye camera (Kyocera Corp., Kyoto, Japan). At the conclusion of the experiment (day 20) the photographs were randomly numbered, mixed, and scored by four different observers, in blinded fashion, by comparing them with a standardized set of photographs with mucositis scores ranging from 0 (no mucositis) to 10 (severe mucositis). Data were collected and mean daily mucositis scores were calculated for each group (Table I). Parametric statistical analysis was performed between groups 2 and 5 and between groups 4 and 6 with the unpaired *t* test. Mean daily mucositis scores (\pm SE) were plotted for all groups to analyze trends in healing by comparing the experimental groups with the control. Daily weights were evaluated to assess the overall health of the animals throughout the experiment. A survival curve was plotted to determine whether any significant differences in unplanned mortality existed between experimental groups and the control.

RESULTS

The presence of EGF significantly modified the frequency and course of mucositis. Hamsters that re-

Table II. Relationship between pump placement and peak mucositis score

Day pump placed	Peak mucositis day
0	11
3	12-13
6	13
7	14-15

ceived EGF had more severe, prolonged mucositis than animals that received placebo. In addition, the timing of pump placement, that is, the initiation of EGF exposure, affected the course of mucositis. For example, hamsters in which 7-day EGF pumps were implanted 1 week after the commencement of chemotherapy (group 2) had more significant mucositis throughout the course of the experiment than animals in which 7-day placebo pumps were implanted (group 5), except for day 11 (Fig. 3). Similarly, the course of mucositis was worse in hamsters in which 14-day EGF pumps were implanted (group 4) compared with animals with 14-day placebo pumps (group 6) (Fig. 4). Delivery of EGF by pumps placed on either day 3 or day 6 failed to alter favorably the course or severity

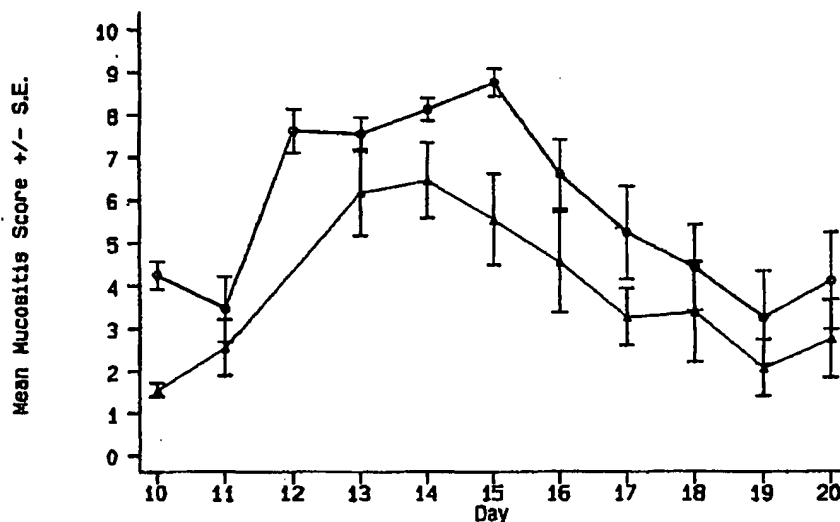


Fig. 3. Comparison of mucositis scores for osmotic pumps containing EGF (O) or placebo (Δ) placed for 7 days. EGF-filled pumps (group 2) were inserted on day 7. Pumps loaded with placebo (group 5) were placed on same day. Pumps placed in both groups operated for 7 days.

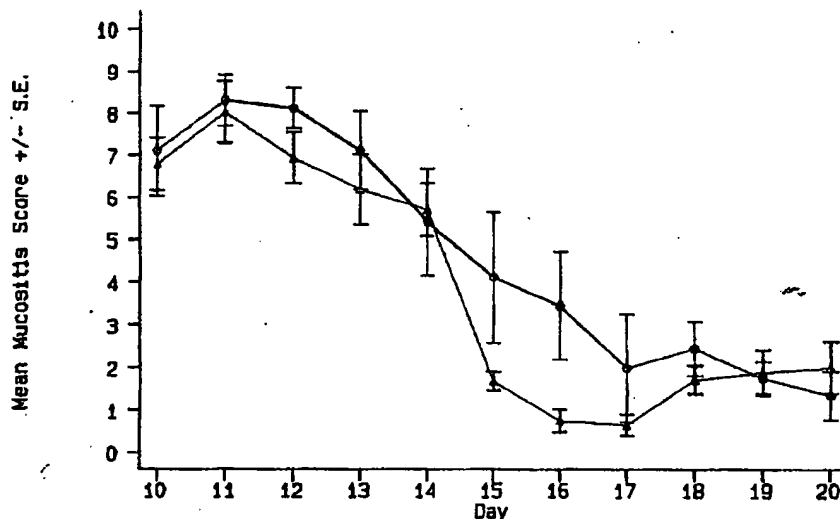


Fig. 4. Comparison of mucositis scores for osmotic pumps containing EGF (O) or placebo (Δ) placed for 14 days. EGF-filled pumps (group 4) were inserted on day 1. Pumps loaded with placebo (group 6) were placed on same day. Pumps placed in both groups operated for 14 days.

of mucositis (Figs. 5 and 6). The earlier the pumps were placed, the more rapid the onset of peak mucositis (Table II). In addition, placement of the pumps early in the experimental period resulted in a more rapid development of mucositis than the placement of pumps 1 week after the start of chemotherapy. Thus animals in groups 2 and 5 had the least severe mucositis as measured on day 10.

As expected, a direct relationship existed between the severity of mucositis and weight loss (Fig. 7). Animals in group 6 had the least mucositis and demon-

strated little fluctuation in weight during the experimental period. In contrast, the prolonged mucositis noted in animals in group 1 resulted in a dramatic weight loss during the experiment. Overall, the onset of weight recovery was noted in all groups at about day 16 to 17.

Survival appeared to be related more to the severity and onset of mucositis than to the presence or absence of EGF. Nonsurgical deaths were first of significance on day 10 and were noted in both EGF and placebo groups (Fig. 8). Hamsters that had

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and might be related to the timing of cytokine application relative to mucositis. Although differences in timing of pump placement affected peak mucositis, in all cases EGF was initiated before optimum mucosal breakdown. Had the application of EGF been delayed until after the stomatotoxic effects had resolved, probably after day 10, it is possible that it might have resulted in a favorable effect on ulcer resolution.

These results also suggest that an effective approach to the prevention of mucositis might be therapy aimed at inhibiting or reducing the rate of mucosal proliferation. We have initiated studies to evaluate this tactic further.

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